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(21) International Application Number: PCT/US92/04781 (22) International Filing Date: 5 June 1992 (05.06.92) (30) Priority data: 712,968 10 June 1991 (10.06.91) US (71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 300 Lakeside Drive, Oakland, CA 94612 (US). (72) Inventor: DENNIS, Edward, A. ; 2731 Glenwick Place, La Jolla, CA 92037 (US). (74) Agents: DREGER, Walter, H. et al.; Flehr, Hohbach, Test, Albritton & Herbert, 4 Embarcadero Center, Suite 3400, San Francisco, CA 94111-4187 (US).		(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC (European patent), MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, RU, SD, SE, SE (European patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: NOVEL PHOSPHOLIPASE A2 INHIBITORS (57) Abstract <p>The present invention provides prodrugs that serve as useful therapeutics for various disease states and conditions mediated by underlying specific hydrolytic enzyme activity. The prodrugs hereof (additionally) impart a physiologically bioactive component thus providing prodrug compounds that are capable of imparting dual effect systemically.</p>		

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NOVEL PHOSPHOLIPASE A2 INHIBITORS

Related Applications

The present invention is related to U.S. Ser. No. _____, filed concurrently with the present
5 application, said concurrently filed application being a continuing application under 35 U.S.C. 120/121 of application Ser. No. 07/399,799, filed 29 August 1989.

The application filed 29 August 1989 is directed to
10 novel hydrolytic enzyme inhibitors/inactivators and substrates functioning as suicide-inhibitory bifunctionally linked substrates (SIBLINKS) useful as 1) therapeutics for pathological disease states or conditions mediated by specific hydrolytic enzyme
15 activity and 2) enzyme substrates for assays specific to hydrolytic enzyme activity. The continuing application of that earlier application, filed concurrently herewith, focuses on the function of the disclosed compounds as inhibitors/inactivators of
20 said hydrolytic enzymes. That distinct use forms the basis of therapeutics and of methods of treatment using such therapeutics. That distinct aspect of the subject matter disclosed in the earlier application is characterized by the novel compounds useful for
25 that embodiment as being physiologically acceptable, and in particular, that the leaving group disclosed, BX, or its component, B, be one that is

physiologically acceptable. Further, the rate of leaving of the BX group is such that, together with the enzyme hydrolysis of moiety AX of said compounds, intramolecular cyclization of the functional residue attends consequent reactivity with the active site of the target hydrolytic enzyme such that it is inhibited/inactivated. That end point can be exploited clinically in the treatment of disease states or conditions that are manifest by the mediated activity of the hydrolytic enzyme.

The present application is directed to a specific subset of such therapeutics, that function as prodrugs.

Field of the Invention

The present invention in all of its aspects utilizes as a fundamental predicate a novel subset of a class of hydrolytic enzyme inhibitors (inactivators)/substrates and their use as prodrugs in the therapeutic treatment of pathological disease states or conditions mediated by specific hydrolytic enzyme activity. These inhibitors function as suicide-inhibitory bifunctionally linked substrates (SIBLINKS) and are characterized as an ensemble of three functional moieties: 1) one recognizable by (an) active site(s) of a given hydrolytic enzyme such that the enzyme functions hydrolytically when contacted with the inhibitor with attendant removal of that moiety, 2) leaving group that is physiologically acceptable and that itself is a physiologically bioactive moiety useful systemically and 3) a remainder moiety linking the first and second that assumes a cyclic configuration after removal of the first and second moieties that may

attend its further reaction with the enzyme active site thus irreversibly inactivating or inhibiting bioactivity of the enzyme through covalent bond formation at said active site(s). IF the
5 inactivating reaction does not take place, as it is not a prerequisite herein, the cyclic residue is otherwise physiologically removed. While, in preferred embodiments, benefits both of the enzyme inactivation and of the physiologically acceptable
10 and bioactive moiety are clinically useful, in all events, availability of the physiologically acceptable and bioactive moiety is a predicate of the present invention.

The novel hydrolytic inhibitors/inactivators of the
15 present invention thus create means for modulating hydrolytic enzyme activity in the control or treatment of various disease states or conditions in which such hydrolytic enzyme activity is implicated. Additionally, as the present compounds can be
20 considered "prodrugs", they also provide physiologically bioactive properties by virtue of the therapeutic effect provided by the physiologically bioactive leaving group that is liberated in the mechanism attending the optional inactivation of the
25 hydrolytic enzyme activity.

Background of the Invention

Considerable background material can be taken from the appropriate section of the above cited earlier applications, and such subject matter is hereby
30 expressly incorporated by reference.

The object of an invention disclosed in the earlier applications was to produce substances that can interfere with disease states or conditions via

molecular interaction of specific hydrolytic enzyme activity on a suicidal inactivation or inhibitor mechanistic level. Based upon that research and study, using phospholipase A₂ as a model, the invention focused on the design of novel hydrolytic enzyme inhibitors (inactivators) functioning via recognition by the active site of such enzymes resulting in inhibition of enzyme functionality. Thus, the inhibitors invited functional suicide of the enzymatic activity.

Summary of the Invention

Taken in its several aspects the present invention stems from the fundamental predicate based upon a novel class of hydrolytic enzyme inhibitors (inactivators) and substrates that function notably as prodrugs. These novel compounds function after recognition and processing by a specific hydrolytic enzyme, in preferred applications inhibiting said enzyme or inactivating said enzyme irreversibly. In the process mechanistically of optionally inhibiting or inactivating said enzyme, a functional moiety is generated that itself is physiologically bioactive thus imparting (additional) therapeutic effect. Preferred means thus form the basis of therapeutics having dual effect and of methods of treatment using such therapeutics. The present invention primarily provides a prodrug that serves as a delivery means of a physiologically acceptable, bioactive drug entity. The present invention further produces associated means germane to such clinically distinct treatment methods.

All of the foregoing aspects and all of their associated embodiments that will be represented as equivalents within the skill of the relevant art are

also included within the ambit and/or interpretation of the present invention.

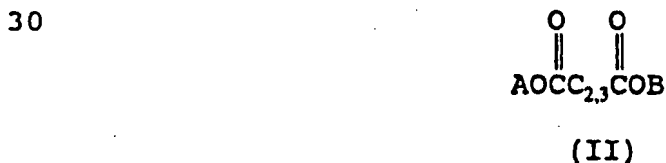
The novel prodrugs of the present invention may be represented by the following generic formula (I):



wherein

- 10 R is an oxygen atom or an imino group,
 each X independently is an oxygen atom, a
 sulfur atom or an imino group,
 A is an enzyme-specific moiety that
 facilitates recognition by and hydrolysis of the bond
15 linking AX with C(0)Y by a target hydrolytic enzyme,
 B is a component of a physiologically
 acceptable leaving group BX that together with the
 enzyme hydrolysis of AX, attends intramolecular
 cyclization of the functional residue, and is itself
20 physiologically bioactive,
 Y is a linker that provides a steric
 environment facilitating intramolecular cyclization
 of said functional residue with optional consequent
 reactivity with the active site of said target
25 hydrolytic enzyme.

More specifically, the novel prodrugs of the present invention, as represented above by formula I, can be represented as a preferred sub-grouping of compounds of the following formula (II);



- wherein each of A and B is as defined above and C_{2,3}
35 represents a linker species having at least two or

three carbon atoms that can be saturated or unsaturated, unsubstituted or substituted.

Further preferred of the class of novel compounds hereof as represented above by Formulas (I) and (II),
5 Group A is preferably selected from a grouping that has a glycerol backbone wherein one oxygen atom is linked to the linker of the above representative compounds; other hetero atoms attached to the glycerol backbone are linked: one, either oxygen,
10 nitrogen or sulfur, to an alkyl or a fatty acid chain, and one, an oxygen, via a phosphodiester or other suitable linkage with a polar group, for example, choline. The fatty acid chain can be a saturated or unsaturated chain that will correspond
15 with the substrate specificity, if any, of the specific hydrolytic enzyme in question.

In a second aspect, the present invention is directed to the method and means for treating a pathological disease state or condition mediated by a specific
20 hydrolytic enzyme activity comprising administering to a subject susceptible to or experiencing said pathological disease state or condition an amount of novel hydrolytic enzyme inhibitor (inactivator) prodrug hereof, sufficient to irreversibly inactivate
25 or inhibit said hydrolytic enzyme activity, and impart additional therapeutic effect, said hydrolytic enzyme inhibitor (inactivator) prodrug being administered in a pharmaceutically acceptable form.

The present invention as defined above in its various
30 aspects includes all associated means and methods in the form of pharmaceutical embodiments, such as formulations and methods for preparing them, pharmaceutical uses, and so forth.

The present invention is described mechanistically in the manner it is presumed to biologically function: however, it shall be understood that the mechanism as such is not necessarily included within the ambit
5 hereof should it actually differ in detail from that proposed. Following the presumed mechanism the functional residue" is" $\begin{array}{c} \text{O} \quad \text{O} \\ \parallel \quad \parallel \\ \text{CYC} \end{array}$.

10 That is, the method of treatment hereof has the endpoint of inhibition and/or inactivation of enzyme activity and additional therapeutic effect attributable to the liberated physiologically bioactive moiety attending the mechanistic action,
15 regardless of the precise mechanism by which such endpoint is manifested in employing the compounds hereof.

Detailed Description of the Invention

All documents referred to herein are hereby expressly
20 incorporated by reference.

The present invention is illustrated by means of a model system whereby particular novel hydrolytic enzyme inhibitors (inactivators) hereof are used in connection with phospholipase A₂. The approach of
25 this invention, as illustrated herein by the model system, can be and is generalized to facilitate the development of various other specific hydrolytic enzyme inhibitors hereof, in assays or treatment regimes for other specific hydrolytic enzymes.

30 Included among such other hydrolytic enzymes are phospholipases, lipases, esterases, proteases, etc. Therapeutic applications for inhibitors hereof for these classes of enzymes arise from conditions such

as inflammation, hypertension, lipid metabolism, obesity, etc.

The essential feature of the compounds hereof is the employment of a bifunctional link to join in a molecular ensemble functionally comprising the necessary structural features required for recognition by a specific hydrolytic enzyme active site(s) and a physiologically acceptable and physiologically bioactive leaving group. In preferred embodiments, the link is a dibasic acid capable of forming a cyclic anhydride. Upon enzymatic hydrolysis of the bond joining the link to the moiety conveying enzymatic specificity, the carboxylate anion of the resulting hydrolysis product is thought to act as a nucleophilic catalyst to cleave the ester bond joining the leaving group and the link, generating a cyclic anhydride. The reactivity of the anhydride with the active site of the enzyme creates a covalent bond between the two thus inactivating, or at least inhibiting, enzymatic activity. The reactivity of the anhydride is sufficiently great that if it should diffuse out of the active site of the enzyme, the overwhelming probability is that it would react with water before encountering another protein.

Thus, mechanistically, upon enzymatic hydrolysis of the bond joining the link to the ensemble conveying enzymatic specificity, the carboxylate anion of the resulting hydrolysis product is thought to act as a nucleophilic catalyst to cleave the bond joining the leaving group and the link. The net result is to generate a reactive cyclic anhydride at the catalytically active site. Acylation by the anhydride of a nucleophilic group of the enzyme irreversibly inactivates the enzyme. The rate of

formation of the anhydride can be modulated by

- 1) adjustment of the pKa of the leaving group, and
 - 2) introduction of alkyl or other substituents on the intervening atoms of the link, or 3) incorporation of
- 5 the intervening linker atoms into a cyclic structure such as an aromatic grouping.

The starting materials bearing such linkers or for constructing such linkers are available in the art - see, e.g., the Aldrich Chemical Co. More detail

10 concerning the chemistry is set forth infra.

The principal advantages for the novel compounds of this invention are general applicability to hydrolytic enzymes and high specificity for particular target enzymes due to 1) compound design

15 and 2) in one embodiment, occurrence of enzymatic acylation principally within the enzyme substrate complex that generated the cyclic compound.

The equivalent mechanistic principles apply where the link is a carbonyl/amide.

- 20 In the design of a moiety A that is specific for a given hydrolytic enzyme, advantage is taken of knowledge of the substrate specificity of the given hydrolytic enzyme. Examples of such can be taken from extant literature and include chymotrypsin,
- 25 lipase, proteases and phospholipase A₂. More detail is provided infra.

Proteases can be subdivided into four major classes reflecting the nature of the catalytic site. Two classes promote hydrolysis of peptide bonds by

30 nucleophilic catalysis entailing the formation of an acyl enzyme intermediate. These are the serine and

cysteine proteases which utilize respectively either the hydroxyl of a serine residue or the thiol of a cysteine residue at the active site to cleave the peptide bond of the substrate. These enzymes will process nonpeptide bonds and accordingly chromogenic assays have been devised. Similarly, this substrate flexibility has allowed a variety of mechanism-based inhibitors to be developed.

The remaining two classes utilize an activated water molecule bound at the active site to cleave a peptide bond. Normally only peptide bonds are processed; consequently, chromogenic assays entailing the release of a dye as a function of enzyme activity have not been as feasible. The two classes are metallo-proteases and aspartic proteases. Good mechanism-based inhibitors for these two classes are unknown reflecting the rigorous criteria for substrate recognition.

In general, the active site of all proteases can be envisaged as lying in a cleft which may have a number of binding pockets to accommodate not only the side chain residues of the amino acid residue that comprise the peptide linkage to be cleaved but also the side chains of the amino acid residues that precede and follow the peptide bond to be cleaved. The high substrate specificity is a reflection not only of the binding requirements in the vicinity of the active site but also of these additional binding sites. Sites that bind to side chain residues that lie toward the N-terminus of the substrate are labeled s_1 - s_n proceeding away from the active site; similarly, sites that bind residues extending toward the C-terminus are labeled s_1' - s_n' . Proteases can either be exopeptidase (cleaves the first or last

peptide bond of the substrate) or endopeptidases (cleaves a peptide bond embedded in the substrate).

To modulate the activity of proteases, the inhibitor must contain appropriate functionality such that "A" occupies the necessary s_1 - s_2 recognition. "Y" and "B" would occupy the s_1 site. Some endopeptidases require occupancy of the s_2 and s_3 sites. In these cases the structure of "Y" must include features which would meet these requirements for substrate recognition. This is most easily accomplished by Y being a substituted aspartic or glutamic acid.

The following is a partial listing of therapeutically useful targets by enzyme class.

Metallo-proteases

- 1) Collagenase, arthritis
- 2) Elastase, emphysema, inflammation
- 3) Angiotensin converting enzyme, hypertension

Aspartic proteases

- 1) HIV protease, AIDS proliferation
- 2) Renin, hypertension
- 3) pepsin, ulcer

Cysteine Proteases

- 1) Cathepsin B, inflammation

Serine Proteases

- 1) Trypsin, pancreatitis
- 2) Granulocyte elastase, inflammation
- 3) Thrombin, coronary infarction

For each of the above enzymes, the design of suicide inhibitors would be guided by known substrate requirements, and secondly, if available, X-ray structural data. For example, renin recognizes the sequence HisProPheHisLeuValIleHis and cleaves the Leu-Val bond. Replacement of the Leu residue with an

aspartic acid residue in which the terminal carboxyl group was esterified with a leaving group "B" would generate a substrate that upon processing would generate a cyclic anhydride which upon acylation of
5 renin could render it inactive. See Barrett & Salvesen Ed. Proteinase Inhibitors Vol. 12 Elsevier, Amsterdam (1986) and Hydrolytic Enzymes Ed. Neuberger & Brocklehurst, Elsevier, Amsterdam (1987).

The chemistry of preparing the novel hydrolytic
10 enzyme inhibitors (inactivators) and substrates hereof is generally known to the skilled organic chemist. For example, where one is employing a dibasic acid in preferred embodiments hereof, both
15 moieties A and B can be attached via usual esterification reactions. The dibasic acid starting material is either known in the art or can be prepared by standard dibasic acidification procedures. See standard organic chemistry and procedure texts.

20
$$\begin{array}{c} \text{O} \quad \text{O} \\ \parallel \quad \parallel \\ \text{AXC-Y-C-XB} \end{array}$$

Given the modular nature of the AXC-Y-C-XB ensemble, the synthetic sequence can be either: 1) reaction of the AXH with an activated dibasic acid followed by
25 activation and reaction with BXH, or 2) synthesis of

$$\begin{array}{c} \text{O} \\ \parallel \\ \text{BXC-Y-CO}_2\text{H} \end{array}$$

utilizing the procedure of Gaetjens et al., J. Amer. Chem. Soc. 82, 5328 (1960), for
30 example, followed by activation and reaction with AXH.

A typical reaction pathway could include heating a mixture of a lysophospholipid, e.g., 1-decanoyl-sn-glycerol-3-phosphorylcholine with an excess of an
35 anhydride, e.g., 2,2-dimethylglutaric anhydride in

methylene dichloride in the presence of a base, e.g., triethylamine. After purification of the product half-acid phospholipid on silica gel, this product would be activated by reaction with an excess of an
5 activator, e.g., oxalyl chloride, in methylene dichloride to produce an acid chloride. This would be subsequently reacted with an excess of the desired BXH group, e.g., p-nitrophenol, as an example where its cleavage could be followed spectrophotometrically
10 and base, e.g., triethylamine. The product in methylene dichloride would then be purified. Alternatively, the BXH group, e.g., p-nitrophenol, could be mixed with an equivalent of base, e.g., sodium hydroxide, and allowed to react with an
15 equivalent of the desired anhydride, e.g., 2,2-dimethylglutaric acid to produce the half-acid. This product can in turn be activated, e.g., with oxalylchloride, and reacted with a lyso phospholipid, e.g., 1-decanoyl-sn-glycerol-3-phosphorylcholine, to
20 produce the desired final product.

Where the novel compounds hereof are selected from those wherein R is NH and/or each X is sulfur or NH, again, standard organic chemistry reactions apply. Briefly, where R is NH, for an example, hydrogen
25 chloride can be bubbled through a methylene chloride solution containing a equimolar mixture of 4-cyanobutyric acid and p-nitrophenol to generate the imino ether. Treatment of this compound with oxalyl chloride in methylene chloride followed by removal of
30 the oxalyl chloride under vacuum and then by addition of moiety AXH and one equivalent of base generates the desired compound.

Where either X is sulfur or nitrogen, the same procedure as described infra for the X = oxygen
35 compounds would be followed except for the

substitution of the appropriate ASH or ANH_2 for AOH
or BSH or BNH_2 for BOH.

Having described the particular model system employed
in the present research for providing the generic
5 class of prodrugs hereof, and having supplied the
methodology for preparing such based upon generally
well known organic chemistry reactions, and having
illustrated a system whereby this model system can be
employed in the case of Phospholipase A_2 , and having
10 supplied information useful to prepare
pharmaceutically acceptable compositions of such
compounds for use in the treatment of implicated
disease states or conditions, the present disclosure
is sufficient to enable one to prepare other
15 prodrugs, methods of treatment and kits, etc., for
their employment in an equivalent pharmaceutically
based regimen. Thus, researchers using extant
literature and techniques and the present concept
shall well enough know how to prepare and design
20 inhibitors (inactivators) of the present invention
for specific hydrolytic enzymes either known or yet
to be discovered. Thus, one would 1) vary the
structural features of the natural substrate to
identify the basic requirements, 2) synthesize a
25 compound containing these features (embodied in
moiety A), 3) covalently join A and Y (the link), and
4) attach B to the basic AY ensemble.

Detailed Description

1. Definitions

30 By the terms relating to the linker depicted above by
Y in the above formulas, is meant a moiety that
serves two functions: It has at each end appropriate
functionality so as to be capable of linkage with
moieties A and B. In further preferred embodiments,

that linkage is via carboxylate functionality. The second requirement is that it contain structural, steric features that favor formation of a cyclic compound upon enzymatic cleavage of the side grouping

5 A and concomitant expulsion of B. In further preferred embodiments, the linker would contain at least two or three carbon atoms, saturated or unsaturated, substituted or unsubstituted. It may be a part of an aromatic arrangement such as is

10 illustrated by a phenyl or naphthalene grouping. The only limitations foreseen are that upon cleavage of side groupings A and B, in situ chemically proximate to the target enzyme, it would intramolecularly bond so as to form a cyclic compound. In the case of the

15 preferred embodiment, the end cyclic compound would be an anhydride (see Figure 1, for example).

By the term referring to a moiety capable of binding to an active site is meant an active site specific moiety that is recognizable by a particular

20 hydrolytic enzyme. In the case of lipases, such a moiety could contain a glycerol backbone where one of the oxygen atoms is linked to the linker and the other two oxygens would be linked to a saturated or unsaturated acyl or alkyl chain appropriate to the

25 enzyme in question. In the case of phospholipase hydrolytic enzymes, one of the two other oxygen atoms would be linked to a phosphodiester having a polar group, for example, choline, ethanolamine, serine, inositol, glycerol, methyl, etc. In the case of

30 other esterases that act on lipids, such as cholesterol esterase, the moiety could contain cholesterol or a derivative.

In the case of proteases, the moiety capable of binding to an active site could be composed of an

35 appropriate amino acid peptide, or analogue,

depending on the substrate requirements of the particular enzyme. For some proteases certain substituents on the linker Y and the leaving group (BX) which may also be an amino acid, peptide or
5 analogue thereof, are also appropriate.

In the case of terms relating to moiety B, there are the requirements: 1) that it be a component of a good leaving group; 2) that, to be used in drug applications where it is a concomitant enzyme
10 inhibitor, the reaction attending intramolecular cyclization of the functional residue be generally rapid relative to diffusion and that it be a component of a physiologically acceptable leaving group; and 3) that it itself be physiologically
15 bioactive.

By the term "modulating" in respect of various disease states or conditions is meant affecting the hydrolytic enzyme activity where such activity is implicated in the onset or continuance or propensity
20 for given disease state or condition symptoms. In the case of preferred embodiments herein, various inflammatory conditions can be alleviated by use of a phospholipase A₂ inhibitor of the present invention so as to reduce or limit the action of said enzyme in
25 the production of products or co-products that either themselves, or after further reactions, induce inflammatory states.

In therapeutic applications, it is essential that the compounds hereof be non-toxic or physiologically
30 acceptable. In particular, the characterization of leaving group BX or its component B, must satisfy this criterion, as a distinct departure from its characterization when used in assay applications where it need only be detectable and measurable.

- Further, in the prodrugs hereof, the B containing leaving group is also physiologically bioactive. Examples of physiologically acceptable, bioactive leaving groups are: Substituted or unsubstituted
- 5 cycloaliphatic or unsaturated (including aromatic) cyclic alcohols, thiols, or imides, such as p-sulfophenyloxy, p-trifluoromethylphenyloxy, p-hydroxytetrafluorophenyloxy, p-halophenyloxy, o,o,p-trimethylphenyloxy, p-acetyloxyphenyloxy,
- 10 p-(trifluoromethyl)methylphenyloxy, p-trimethylaminophenyloxy, p-cyanophenyloxy, o-carboxyphenyloxy, o-carboxy-p-aminophenyloxy, N-(acetylamino)phenyloxy, 2-(1-carboxyethyl)-naphth-6-yl-oxy, and so forth.
- 15 See also, for example, Kirby, Adv. Phys. Org. Chem. 17, 183-278 (1980). Physiological acceptability can be determined as well in accord with federally regulated clinical studies.

- By "lower alkyl" or "alkyl" is meant all isomers
- 20 comprising 1 to 4 carbon atoms, inclusive.

2. Examples

Preparation of Model phospholipase A₂ Specific Inhibitor Prodrugs

Materials

- 25 All lysophospholipids, 1,2-dipalmitoyl-sn-glycero-3-phosphorylcholine (DPPC), and 1,2-dicaproyl-sn-glycero-3-phosphorylcholine (DCPC) are purchased commercially. All cyclic anhydrides except 2,2-dimethylsuccinic anhydride are obtainable from
- 30 Aldrich Chemical Co. The latter is prepared by treating dimethyl-succinic acid (Aldrich) with a 3-fold excess of trifluoroacetic anhydride in CH_2Cl_2 for 2 hours, removing the volatiles, and using the

residual solid without further purification. All solvents and reagents are of reagent quality.

PLA₂ obtained from cobra venom (N. naja naja) was purified as described in Hazlett et al., Toxicon 23,
5 457 (1985).

Preparation of Physiologically Acceptable, Bioactive
SIBLINKS

To 42 mg (0.1 mmol) of 1-decanoyl-sn-glycerol-3-phosphorylcholine in 3 ml of CH₂Cl₂, is added 60 mg
10 (0.43 mmol) of 2,2-dimethylglutaric anhydride followed by 50 μ l (0.35 mmol) of Et₃N. The reaction is heated for 24 hours at 45°C. If the reaction is not complete by TLC analysis (1:10:22 H₂O/MeOH/CHCl₃, using Analtech Silica Gel G-250 glass plates with UV
15 indicator visualized with molybdate spray), an additional 30 mg (0.21 mmol) of anhydride with 40 μ l (0.28 mmol) of Et₃N are added and the reaction heated for an additional 10 hours until all starting lipid is consumed. After removal of all volatile
20 components, the residue is leached three to four times with 10-ml portions of dry Et₂O to remove unreacted anhydride and triethylamine glutaric acid salts. The remaining crude half-acid phospholipid after evacuation at 0.1 torr is converted to the acid
25 chloride upon treatment with 2 ml of CH₂Cl₂, containing 0.3 ml of oxalyl chloride for 5 hours at 20°C. Alternatively the half-acid phospholipid is isolated by washing with 0.1 N HCl the crude acylation product dissolved in 2:1 CHCl₃/MeOH. Pure half-acid
30 phospholipid is obtained after chromatography on silica gel using 1:4:30:65 HOAc/H₂O/MeOH/CHCl₃, as the eluant.

The acid chloride is separated from oxalyl chloride by removal of the volatiles in vacuo followed by two cycles of dissolution in 2 ml of dry benzene and evaporation. A solution of 65 mg (0.43 mmol) of p-
5 (N-acetylamino)-phenol and 60 μ l (0.42 mmol) of Et₃N in 4 ml of CH₂Cl₂ is added to a 1-ml CH₂Cl₂ solution of the acid chloride. If necessary, additional phenol and amine are added to ensure that an excess of p-(N-acetylamino)-phenoxide is present. After standing
10 overnight at 20°C, the volatiles are removed and the residue taken up in 5 ml of 2:1 CHCl₃/MeOH and washed with 4 ml of 0.1 N HCl. The solvent is removed, and the residue containing phospholipids is chromatographed on silica gel using 10-33% MeOH/CHCl₃,
15 as the eluant to give the desired phospholipid.

Pure samples are obtained after HPLC chromatography using anhydrous MeOH to elute the lipid from a Brownlee C₁₈ column.

Other compounds falling within the scope of the
20 present invention are prepared following analogous procedures with appropriate starting compounds as set forth in the following Table:

Table I

Starting Compounds			
	For B Moiety	For Y Moiety	For A Moiety
5	salicyclic acid	glutaric anhydride	1-decanoyl-lyso-PC*
	salicyclic acid	glutaric anhydride	1-decanoyl-lyso PE (phosphatidylethanolamine)
	salicyclic acid	glutaric anhydride	1-decanoyl-lyso PG (phosphatidylglycerol)
	salicyclic acid	glutaric anhydride	1-decanoyl-lyso PS (phosphatidylserine)
10	p-(N-acetylamino)phenol	pimelic anhydride	1-decanoyl-lyso PC
	p-(N-acetylamino)phenol	pimelic anhydride	1-decanylthio-lyso PC
	p-(N-acetylamino)phenol	2-methylsuccinic anhydride	1-decanylthio-lyso PG
	p-(N-acetylamino)phenol	2,2-dimethylsuccinic anhydride	1-decanylthio-lyso PE
	p-(N-acetylamino)phenol	1,2-dimethylsuccinic anhydride	1-decanylthio-lyso PS
	p-(N-acetylamino)phenol	3,3-dimethylsuccinic anhydride	1-decanylamino-lyso PC
15	p-trifluoromethyl phenol	maleic anhydride	1-decanoyl-lyso-PC
	p-trifluoro-methyl phenol	norbornyl anhydride	1-decanoyl-lyso-PC
	p-bromo phenol	norbornyl anhydride	1-decanoyl-lyso-PC
	p-acetyloxy phenol	glutaric anhydride	1-decanoyl-lyso-PC
	2-(6-hydroxynaph-2- yl)propionic acid	glutaric anhydride	1-decanoyl-lyso-PC
20	indomethacin	naphthylene-2,8-di- carboxylic acid anhydride	1-decanoyl-lyso-PS
	tolmetinsodium	benzene-1,2- dicarboxylic acid anhydride	1-hexanoyl-lyso PC
	salicylic acid	benzene-1,2- dicarboxylic acid anhydride	1-hexanoyl-lyso PC

* 1-decanoyl-2-lysophosphatidylcholine (full name 1-decanoyl-sn-glycero-3-phosphorylcholine)

Preincubation Conditions

SIBLINKS hereof are purified by HPLC SIBLINKS vesicles are prepared by sonicating 2-4 mg of phospholipid in 1 ml of 100 mM KCl using four 30-s pulses of a MSE 100-watt sonicator.

5 The resulting

SUBSTITUTE SHEET

solution is centrifuged (25 min. at 9,500 x g, 4°C). The vesicles after separation from the pellet are analyzed for free BX leaving group. To minimize slow hydrolysis of the aryl ester, the vesicles are stored at 4°C. Further purification of the above vesicle preparation by ultracentrifugation (4 hours at 50,000 x g, 4°C) does not significantly alter inhibition time courses.

Standard preincubation conditions utilizes 100 µM SUBLINKS as sonicated vesicles with PLA₂ in 20 mM Tris-HCl (pH 8.0), 10 mM CaCl₂, and 100 mM KCl at 20°C. The same conditions are used for preincubation with mixed micelles except for the presence of TRITON X-100®. The concentration of N. naja naja PLA₂ is 0.37 µM (5 µg/ml). The preincubation concentrations of the PLA₂s from other sources ranges from 5 to 20 µg/ml.

Residual Activity Determination

To determine the amount of residual enzyme activity remaining after preincubation, a titrametric assay is employed in which 5-20 µl of the preincubation solution is added to 1.7 ml of the substrate solution containing 10 mM CaCl₂ at 40°C. Thus, the preincubation mixture is diluted so activity toward residual SIBLINKS is negligible, and activity toward added substrate is maximized. PLA₂s obtained from N. naja naja and bee venoms are routinely assayed with 5 mM DPPC in mixed micelles with 20 mM TRITON X-100®. The assay utilizes 50 ng of protein. Deems et al., Methods Enzymology 71, 703 (1981). Porcine pancreatic PLA₂ is assayed titrametrically with 100 ng of protein/assay and an egg lecithin/sodium deoxycholate mixture as substrate. Nieuwenhuizen et

al., Methods Enzymology 32b, 147 (1974).
C. Adamanteus and C. atrox PLA₂s are assayed
titrametrically using 70 ng of protein/assay with the
same DPPC/TRITON X-100® assay described above for N.
5 naja naja except for the addition of 1 mg/ml of
bovine serum albumin. Pluckthun et al., J. Biol.
Chem. 260, 11099 (1985). Residual activities
(percent) are calculated from the mole of base
consumed titrametrically relative to a PLA₂ solution
10 preincubated under the same conditions in the absence
of the SIBLINKS.

SIBLINKS Inhibition

The reaction is simultaneously monitored during
preincubation of PLA₂ with SIBLINKS vesicles.
15 Titrametric assays, as described above, reveals the
amount of residual enzymatic activity from which the
number of mole of PLA₂ inactivated can be calculated.
To ascertain maximum inhibition, preincubations are
continued for 24 hours or less if no further loss of
20 activity with time occurs within experimental error.
The partition ratio (P) of the number of mole of
SIBLINKS hydrolyzed per mole of enzyme inactivated is
calculated using independent determinations of mole
of B leaving group released and mol of PLA₂
25 inactivated. P values are calculated several times
during the time course of inactivation studies. P is
essentially constant between 20 and 70% inactivation.
The value of P for a specific SIBLINKS is an average
of the three or four values measured during the
30 determination of each inactivation time course.

Cyclic Anhydride Inhibition

The following procedure is utilized to measure PLA₂ inhibition by cyclic anhydrides. A CH₂Cl₂ solution of the anhydride is evaporated under an N₂ stream.

- 5 Immediately, 400 µl of the appropriate PLA₂ in 20 mM Tris-HCl (pH 8.0), 10 mM CaCl₂, and 100 mM KCl at 20°C is added followed by vortexing to ensure rapid mixing. Aliquots are assayed titrimetrically after 5 min; no further change in activity is observed after
10 an additional 1-2 hours.

Preparative Scale Inhibition of PLA₂

- When large amounts of PLA₂ inhibited by cyclic anhydride are needed, the following procedure is employed. To 0.5-0.9 ml of buffer (0.7 M Tris-HCl,
15 pH 8.0) containing 0.15-0.2 mg of PLA₂ is added 6.5-9 mg of cyclic anhydride 26. After vortexing and standing for 1 hour, a second portion of anhydride is added to ensure that maximum inhibition was obtained. The suspension is applied to a Pharmacia LKB
20 Biotechnology Inc. G-25 PD-10 column that was preequilibrated with buffer (10 mM K₂HPO₄, pH 8), and the protein is eluted with the same buffer. Similar conditions are employed to obtain PLA₂ inhibited by SIBLINKS except for a 20 hour preincubation with 500
25 µM SIBLINKS vesicles and the inclusion of 10 mM CaCl₂ and 100 mM NaCl. The 0.5 ml chromatographic fractions are analyzed for protein and B leaving group ester; only protein fractions free of the SIBLINKS are utilized.

Hydroxylamine Studies

The following procedure is employed for hydroxylamine treatment. The appropriate amount of a freshly prepared stock solution of 50mM $\text{NH}_2\text{OH HCl}$ in 1 M

5 Tris-HCl (pH 8.0) is added to the PLA_2 solution to a final concentration of 5mM. After vortexing and before assaying titrametrically for PLA_2 activity, the solution is allowed to stand 1-2 hours at 20°C. This reaction is performed in a closed vial to

10 minimize NH_2OH oxidation.

Assays

All assays may be measured in 0.4 ml buffer (10mM Tris-HCl, pH8, 10 mM CaCl_2 , and 100 mM KCl).

One may plot specific activity for 20ng phospholipase

15 A_2 obtained from cobra venom (*Naja naja naja*) as a function of the concentration of SIBLINKS hereof in 3.2:1 mixed micelles of Triton X-100 and phospholipid at 40°C. See Dennis, J. Lipid Research 14, 152 (1973) and Deems and Dennis, Methods in Enzymology

20 71, 703 (1981).

One may plot initial velocities (expressed at Δ O.D. at $\lambda = 400$ nm in 20 sec.) observed with phospholipase A_2 (specific activity 1470 $\text{Mmol min}^{-1}\text{mg}^{-1}$ which is linear with protein concentration from 0.5ng to 100ng

25 using 1.8:1 using Triton/phospholipid mixed micelles at 40°C containing 0.4 mM of SIBLINKS compound.

The hydrolysis reaction rate is a function of the ratio of mole fraction of substrate in the Triton/phospholipid mixed micelle; the rate

30 diminishes three-fold as the surface ratio increases from 1.6:1 to 3:1 to 4.5:1 to 7:1. Unilamellar

vesicles (SUVs) prepared by sonication of prodrug compound followed by centrifugation, are readily hydrolyzed by phospholipase A₂; for a 400 μ M solution of SUV's, V was 265 μ mol/min/mg as compared to 550 measured for 400 μ M of compound 1a in 3.2:1 Triton mixed micelles.

The time courses for inactivation of phospholipase A₂ obtained from cobra venom by preincubation of prodrugs hereof are obtained by 1) preincubate a 260:1 mixture of inhibitor 1 to PLA₂ in 1 ml solution containing 5 μ g/ml of PLA₂, 100 μ M vesicles of prodrug 1a-1e, 20 mM Tris-HCl, pH 8, 10 mM CaCl₂ and 0.1 M KCl at 20°C, 2) measure titrimetrically the hydrolysis rate initiated by addition of a 20 μ l aliquot of the above solution to 1.7 ml of 40°C assay medium containing 5 mM 1,2-dipalmitoyl phosphatidylcholine, 10 mM CaCl₂ and 20 mM Triton X-100.

The reader is directed to literature extant that supply relevant details as to specific, assays in measuring activity herein, and to devising pharmaceutically acceptable compositions and methodology for the efficacious treatment of disease states, having supplied herein the essence of the present invention for essentially participating in such clinical endeavors. For example, see U.S. patents 4826958, 4833152, 4616089, 4788304, 4447445, and W086/06100 (23 October 1986).

Drug entities prepared as described above for specific target hydrolytic enzyme inhibition and/or inactivation are compounded in accord with known techniques to produce useful pharmaceutical compositions that are pharmaceutically acceptable for appropriate administration in the treatment of

pathological conditions or disease states manifested etiologically by such hydrolytic enzyme activity.

Such drugs are tested for safety, dose response and efficacy in humans as per federal regulations.

5 Ordinary studies conducted pursuant to those regulations shall determine the safety and efficacious dose regimens appropriate in the circumstances for the treatment of the particular disease state of concern. The attendant clinical
10 studies are in the area of routine experimentation generally within the ken of the art-skilled. These drugs are administered via standard formulations to patients with such disease states, again either topically, orally, parenterally, rectally, alone or
15 in combination, at regular intervals or as a single bolus, or as a continuous infusion, and so forth.

For example, a typical pharmaceutical composition containing the active compound hereof together with an appropriate pharmaceutically acceptable carrier
20 entity(ies) may be in the range of about 0.1 mg to about 500 mg per dose, or about 1 microgram to about 7 mg per kilogram of body weight. Such amount would be considered "an amount sufficient to inactivate or inhibit the activity of a given hydrolytic enzyme".
25 Again, the end-point of such administration would be the inhibition or inactivation of the given hydrolytic enzyme manifested by an alleviation of the symptoms associated with the disease. The regulatory protocols necessary to produce marketable drug
30 entities provide the exact dosage and the details of the "pharmaceutically acceptable form" of a compound of this invention.

The information contained in the part hereof supra entitled "Assays" are materials and methods and

results of in vitro studies using certain of the compounds hereof as models for the testing of inhibition and/or inactivation of a particular model hydrolytic enzyme. These protocols and results are
5 believed to be translatable within the routine experiment of the art-skilled to related enzymes of human origin, and into an animal, and hence, a human being. As mentioned above, confirmation of such translation into these in vivo systems would be
10 readily measurable by the end point of the mechanism believed operative for the compounds of the present invention that are physiologically acceptable. Thus, in such an in vivo system, if the organism exhibits alleviation of symptoms associated with a given
15 disease state that is known to be linked to a particular hydrolytic enzyme against which the test compound hereof is effected, then activity in such a biosystem with such test compound can be presumed in line with and consequential to the corresponding in
20 vitro tests, as provided above.

The foregoing description details specific methods that can be employed to practice the present invention. Having detailed specific methods initially used to characterize, prepare and use the
25 inhibitors (inactivators) and substrates hereof, and further disclosure as to specific model systems, those skilled in the art will well enough know how to devise alternative reliable methods for arriving at the same information and for extending this
30 information to other hydrolytic enzyme systems. Thus, however detailed the foregoing may appear in text, it should not be construed as limiting the overall scope hereof; rather, the ambit of the present invention is to be governed only by the
35 lawful construction of the appended claims.

WHAT IS CLAIMED IS:

1. A method of treating a pathological disease state or condition mediated by specific hydrolytic enzyme activity, comprising administering to a
5 subject susceptible to or experiencing said pathological disease state or condition an amount sufficient to inactivate or inhibit the activity of said hydrolytic enzyme of a pharmaceutically acceptable form of a compound of the formula:



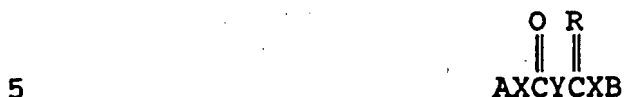
wherein

- R is an oxygen atom or an imino group,
15 each X independently is an oxygen atom, a sulfur atom or an imino group,
A is an enzyme-specific moiety that facilitates recognition by and hydrolysis of the bond linking AX with C(0)Y by a target hydrolytic enzyme,
20 B is a component of a physiologically acceptable leaving group that, together with the enzyme hydrolysis of AX, attends intramolecular cyclization of functional residue, and itself is physiologically bioactive,
25 Y is a linker providing a steric environment facilitating intramolecular cyclization of said functional residue with optional consequent reactivity with the active site of said target hydrolytic enzyme.

- 30 2. The method according to Claim 1 for treating inflammation through inactivation of Phospholipase A₂ activity.

3. The method according to Claim 1 or 2 wherein, in said compound, A is a moiety that facilitates recognition by and hydrolysis of the bond linking AX with C(O)Y by phospholipase A₂.
- 5 4. The method according to Claim 1 or 2 wherein, in said compound, AX is 1-decanoyl-sn-glycero-3-phosphorylcholine.
5. The method according to Claim 4 wherein, in said compound, Y is an alkylene or alkenylene
10 optionally substituted with one or more lower alkyl groups.
6. The method according to Claim 5 wherein, in said compound, Y is n-propylene.
7. The method according to Claim 5 wherein, in
15 said compound, Y is 1,1-dimethyl-n-propylene.
8. The method according to Claim 5 wherein, in said compound, Y is 2,2-dimethyl-n-propylene.
9. The method according to Claim 5 wherein, in said compound, Y is ethylene.
- 20 10. The method according to Claim 5 wherein, in said compound, Y is 1,1-dimethylethylene.
11. The method according to Claim 4 wherein, in said compound, BX is p-(N-acetylamino)-phenyloxy.
12. The method according to Claim 11 wherein, in
25 said compound, BX is o-carboxyphenyloxy.
13. A method for preparing a therapeutically useful composition which comprises compounding a

pharmaceutically acceptable form of a compound of the formula:

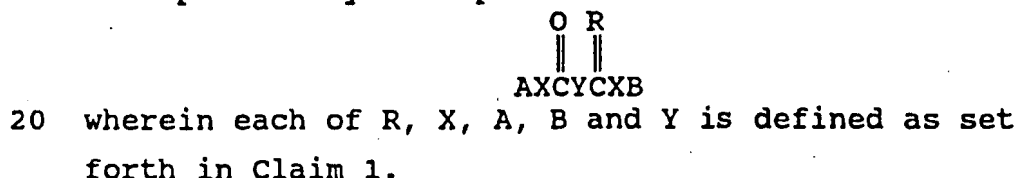


wherein each of R, X, A, B and Y is defined as set forth in Claim 1.

14. A method according to Claim 13 wherein said therapeutic composition is useful for the treatment
10 of inflammation through inactivation of phospholipase A₂ activity.

15. A method according to Claim 13 including the additional step of using said therapeutic composition in a therapeutic regimen in man.

15 16. A method which comprises using therapeutically a compound of the formula



17. The method according to Claim 16 wherein said therapeutic use is for treatment for inflammation through inactivation of phospholipase A₂ activity in
25 vivo.

18. A compound of the formula:



R is an oxygen atom or an imino group,
each X independently is an oxygen atom, a sulfur atom or an imino group,

A is an enzyme-specific moiety that facilitates recognition by and hydrolysis of the bond linking AX with C(O)Y by a target hydrolytic enzyme,

B is a component of a physiologically acceptable leaving group that, together with the enzyme hydrolysis of AX, attends intramolecular cyclization of functional residue, and itself is physiologically bioactive,

Y is a linker providing a steric environment facilitating intramolecular cyclization of said functional residue with optional consequent reactivity with the active site of said target hydrolytic enzyme.

19. A compound according to Claim 18 wherein BX is a radical of a compound of a class selected from cycloaliphatic and unsaturated cyclic alcohols, thiols or imides.

20. A compound according to Claim 19 wherein BX is o-carboxyphenyloxy.

21. A compound according to Claim 20 wherein AX is 1-decanoyl-sn-glycero-3-phosphorylcholine.

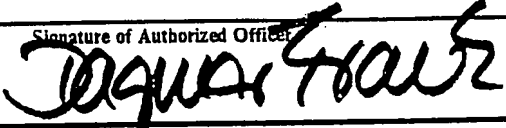
22. A compound according to Claim 21 wherein R is an oxygen atom and Y is n-propylene.

23. A compound according to Claim 21 wherein R is an oxygen atom and Y is 1,1-dimethyl-n-propylene.

24. A compound according to Claim 21 wherein R is an oxygen atom and Y is 2, 2-dimethyl-n-propylene.

25. A compound according to Claim 21 wherein R is an oxygen atom and Y is ethylene.

26. A compound according to Claim 21 wherein R is an oxygen atom and Y is 1,1-dimethylethylene.
27. A compound according to Claim 18 wherein R is an oxygen atom and Y is 1,1-dimethylethylene.
- 5 28. A compound according to Claim 27 wherein AX is 1-decanoyl-sn-glycerol-3-phosphorylcholine.
29. A compound according to Claim 28 wherein X in BX is an oxygen atom.
30. A compound according to Claim 28 where BX is
10 o-carboxyphenyloxy.
31. A compound according to Claim 28 wherein BX is p-(N-acetylamino)-phenyloxy.
32. A compound according to Claim 28 wherein BX is 2-(1-carboxyeth-1-yl)-naph-6-yloxy.
- 15 33. A compound according to Claim 28 wherein BX is o-carboxy-p-aminophenyloxy.

I. CLASSIFICATION OF SUBJECT MATTER (If several classification systems apply, indicate all)		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl.5 A 61 K 31/00 A 61 K 31/685		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl.5	A 61 K C 07 F	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ^o	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	EP,A,0300397 (HOECHST-ROUSSEL) 25 January 1989, see page 8, line 12 - page 10, line 13	18-33
Y	---	1-17
Y	EP,A,0331167 (BOEHRINGER MANNHEIM) 6 September 1989, see claims; page 3	1-17
A	-----	18-33
<div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p>^o Special categories of cited documents : ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
22-09-1992	20. 10. 92	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE		

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9204781
SA 61366

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 14/10/92
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0300397	25-01-89	AU-A- 1970288	27-01-89
		JP-A- 1047792	22-02-89
		US-A- 5030733	09-07-91
EP-A- 0331167	06-09-89	DE-A- 3807123	14-09-89
		AU-A- 3094389	07-09-89
		DE-A- 5890186	27-08-92
		JP-A- 2003662	09-01-90
		US-A- 5091527	25-02-92